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14. ABSTRACT:

In the past funding year, we characterized a novel serine/threonine p21 activated protein kinase 6 (PAK6) as a key signal mediator in regulating non-genomic AR signal transduction within membrane-associated caveolin/raft domain. We found that in prostate cancer cells androgen stimulation activates PAK6. Consequently, PAK6 activation promotes prostate cancer cell motility. Ectopic expression of constitutive active PAK6 in prostate cancer LNCap cells promotes ancharage-independent soft agar colony growth. Our results support the hypothesis set fort from our original proposal that AR signaling from raft to regulate its non-genomic function

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DOD Progress Report:

Caveolin-1 Modulates Androgen Receptor Signaling in Advanced Prostate Cancer

Introduction:

The underlying mechanism of the progression of prostate cancer to hormone-independent disease is poorly understood. Neoexpression of caveolin-1, a scaffold protein associated with caveolae membrane microdomains, has been shown to correlate with hormone resistance and metastasis in both human and mouse prostate cancer models (Nasu et al., 1998; Yang et al., 1999). We find that overexpressing caveolin-1 in human prostate cancer cells positively regulates androgen receptor transactivation activity. We identify that modulating caveolin expression levels dramatically alters the sensitivity of AR to androgen stimulation in cellular models (Lu et al., 2002). We hypothesize that caveolin-1 scaffolding signal complex plays a regulatory role in AR activation pathway. Our specific aims are: (1) Mapping the submolecular domains required for AR and caveolin interaction, (2) Functional and biochemical characterization of the AR/caveolin interaction, (3) Characterization of the physiological role of caveolin-1 overexpression in AR signaling of prostate carcinoma cell, and (4) Evaluating the effect of caveolin scaffolding domain CSD peptide in prostate cancer PC3 tumor growth in vivo.

Body:

In the current budget year, we have partially characterized the biological role of AR-PAK6 interaction in the context of androgen receptor mediated signaling as partial fulfillment of Aim 3. We determined that in prostate cancer cells androgen receptor mediates androgen stimulated PAK6 activation. Furthermore, this activation leads to increased prostate cancer cell motility.

(1) AR-mediated PAK6 activation in response to androgen stimulation. Although PAK6 is identified as an AR interacting protein, it does not function as a co-factor in AR transcriptional activation. To define the role of this interaction physiologically, HEK293 cells were transiently co-transfected with AR and PAK6 expression vectors followed by androgen stimulation. PAK6 kinase activities were determined by an in vitro kinase assay on the immunoprecipitated PAK6 from each experimental group. As shown in Figure 1A, an in vitro kinase assay showed activation time course of PAK6 in response to androgen stimulation. On the other hand, in AR negative PC3 cells, androgen stimulation does not activate PAK6 kinase activity (Figure 1B). These results underscore the role of AR in mediating androgen-stimulated PAK6 activation.

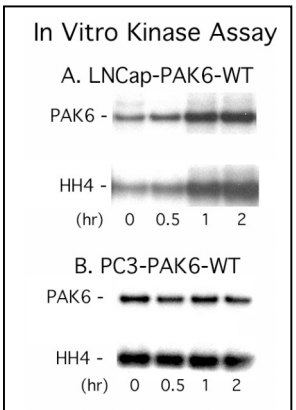

Figure 1

(2) Activated PAK6 promotes prostate cancer cell motility. As described previously, PAK1 was shown to modulate the cytoskeletal reorganization and cell motility in human breast cancer cells. To determine the physiological consequences of PAK6 activation in prostate cancer cells, we generated two functional PAK6 mutants that are either without kinase activity ("kinase dead"), by substituting the conserved lysine residue in the ATP binding cassette within kinase domain with a methionine (K436M), or a constitutive active mutant, by substituting a conserved serine residue from kinase domain VIB with an asparagine (S531N). LNCap cell lines stably expressing WT, K436M and S531N PAK6 were established. The result of an in vitro kinase assay of the immunoprecipitated PAK6 wt and mutants (Figure 2) demonstrates the nullified kinase activity of kinase dead mutant (K436M) as well as extremely high activity of the constitutive active mutant (S531N). Cell motility test was carried out with a transwell assay. The transwell migration assay measures directional cell migration toward an immobilized chemical gradient using a modified Boyden chamber, in this case fibronectin coated membrane is used, by seeding cells on top side of the membrane-well and measuring migrated cells from the other side of the membrane. As shown in Figure 3, cells expressing PAK6-S531N exhibited the highest motility even in the absence of androgen stimulation. LNCap-PAK6WT cells migrated appreciably more than the parental LNCap cells when treated with androgen. In LNCap-K436M cells, the overall transwell migration was basically abrogated as compared with other experimental groups.

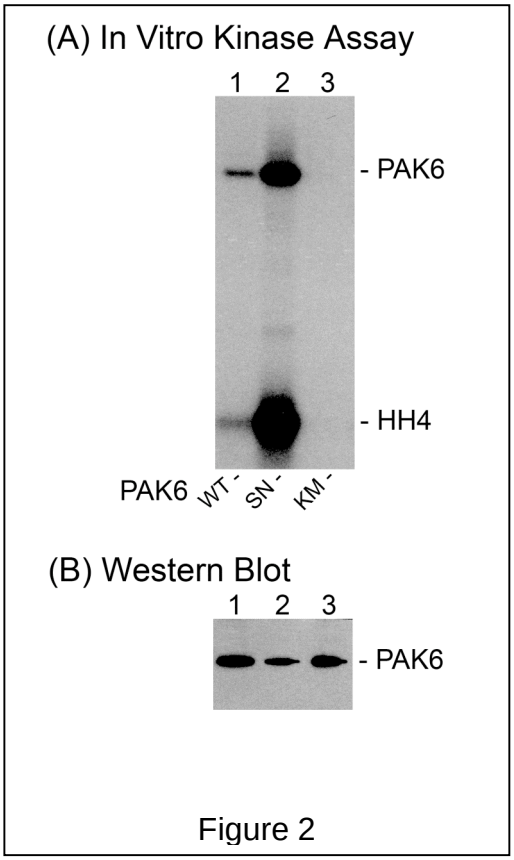

Figure 2

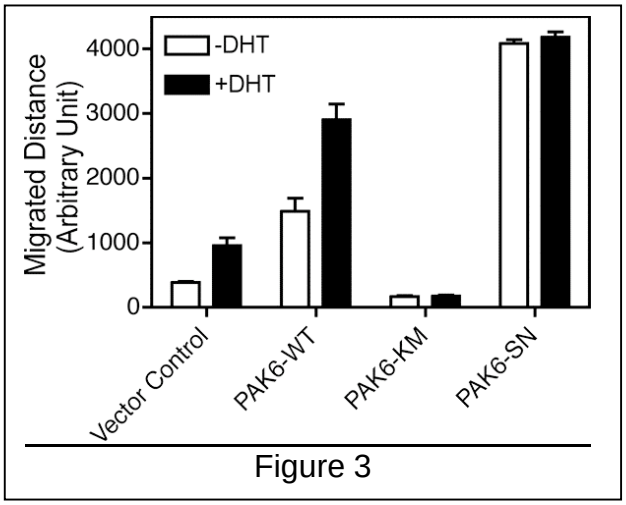

Figure 3

Key Research Accomplishments:

1. We determined that in prostate cancer cells androgen receptor mediates androgen stimulated PAK6 activation.

2. Androgen stimulated PAK6 activation promotes prostate cancer cell motility.

Reportable Outcomes:

1. Publications:

   Freeman MR, Cinar B and Lu ML. Membrane rafts as potential sites of nongenomic hormonal signaling in prostate cancer. Trends Endocrinol. Metabol. Rev., 2005, 16(6): 273-9. (Appendix 1)

2. Cell Lines:
   LNCap cells stably expressing PAK6 wt or functional mutants

Conclusion:

In summary, we identified and preliminarily characterized a novel serine/threonine p21 activated protein kinase 6 (PAK6) as the key signal mediator in regulating AR signal transduction within caveolae/raft domain. Overall, our results established a biochemical basis on the notion that caveolin expression is associated with prostate cancer progression. The "neoexpression" caveolin in prostate cancer progression represents a gain of function event in cancer survival. These results illustrate the important role of AR non-genomic effect in response to androgen stimulation.

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2. Lee SR, Ramos SM, Ko A, Masiello D, Swanson KD, Lu ML, Balk SP. Mol. Endocrinol. **16**:85 (2002)

Appendices:

Review Article:

Michael R. Freeman, Bekir Cinar, and Michael L. Lu. Membrane rafts as potential sites of nongenomic hormonal signaling in prostate cancer. *TRENDS in Endocrinology and Metabolism*, Vol. 16, No. 6. August 2005.

Review TRENDS in Endocrinology and Metabolism Vol.16 No.6 August 2005

Membrane rafts as potential sites of nongenomic hormonal signaling in prostate cancer

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Recent evidence indicates that nuclear receptors for steroid hormones can signal by nongenomic mechanisms that operate independently of their transcription function. These signal-transduction processes occur within seconds to minutes after initiation with agonist and involve interactions between nuclear receptors and other signaling proteins in the cytoplasm and at membrane surfaces. This review provides an overview of published information on possible nongenomic activities of the androgen receptor (AR) and other nuclear receptors, focusing on the potential involvement of these processes in prostate cancer. We discuss the hypothesis that the cholesterol-rich lipid-raft compartment(s) of cancer-cell membranes might provide privileged sites for nongenomic signals mediated by the AR.

Introduction

Prostate cancer (PCa, see Glossary) is a leading cause of premature death in Western countries [1]. An estimated 189 000 men were diagnosed with PCa in 2002 in the USA and an estimated 30 200 died from this disease [2]. There is no effective therapy for disseminated PCa. Late-stage patients are managed primarily in a palliative manner and experience considerable morbidity. After many decades, androgen-ablation remains the principal treatment paradigm for PCa that is not confined to the primary site. However, relapse from hormonal therapy is almost certain and most patients with metastases progress to end-stage disease regardless of treatment strategy. The lack of progress on clinical options for PCa patients reflects our poor understanding of the molecular and cellular mechanisms that underlie disease etiology and progression. In this review, we summarize new findings on the potential role of classical steroid hormone receptors, such as the androgen receptor (AR), as mediators of nongenomic signaling mechanisms. We propose that an attractive cytosolic location for such processes is within the cholesterol-rich 'lipid raft' membrane fraction, which is known to sequester signaling partners in multiple pathways.

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Androgens and prostate cancer

PCa is considered a 'hormone-dependent' disease because the prostate requires testicular androgens for its secretory function and PCa cells retain this sensitivity to androgen. Androgens are believed to be the primary soluble mediators of growth and homeostatic survival of prostate epithelial cells *in situ* [3]. PCa cell-growth and survival pathways respond decisively to androgen, which indicates that this class of steroids is a fundamental mediator of PCa cell physiology. There is an extensive literature on this topic and most investigators believe that androgenic signaling is important for tumor growth and disease progression in prostatic malignancy. Nevertheless, aggressive PCa is considered generally to be 'androgen-independent' because of the uniform failure of therapies that are designed to block androgenic signaling. The mechanisms by which PCa progresses from an androgen-dependent to an androgen-independent disease are not understood.

The principal molecular target for androgens is the androgen receptor (AR), which is a member of the nuclear-receptor family of ligand-dependent transcription factors. AR binds to specific chromatin regions in concert with other transcriptional regulators and controls the expression of many target genes [4]. Although normally AR function is highly dependent on the availability of

Glossary

Akt, protein kinase B
AR, androgen receptor
CSD, caveolin scaffolding domain
E_2, 17β-estradiol
eNOS, endothelial nitric oxide synthase
EGFR, epidermal growth factor receptor
ERα/β, estrogen receptor α or β
ERK, extracellular signal regulated protein kinase
hCG, human chorionic gonadotrophin
IGF-1R, insulin-like growth factor-1 receptor
IL-4, -6, interleukin-4 and -6
LBD, ligand binding domain
MAP kinase, mitogen-activated protein kinase
MNAR, modulator of nongenomic activity of estrogen receptor
mTOR, mammalian target of rapamycin
PCa, prostate cancer
PI-3K, phosphoinositide 3-kinase
PR, progesterone receptor
pTyr, phosphotyrosine
SH2/SH3, Src-homology domain 2 or 3
STAT3, signal transducer and activator of transcription-3

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sufficient androgens to bind directly to and activate the AR, many independent lines of evidence indicate that the AR is likely to play a major role in androgen-independent disease (i.e. in the absence of significant levels of hormone [5]). The mechanisms underlying AR activation by the extremely low levels of androgen present in the castrated condition, and how 'ligand-independent' activation of the AR occurs are only beginning to be revealed. The changes in androgen signaling that occur in PCa are diverse, and include alterations in either the availability or activity of co-activators and repressors of the AR [4,6,7], somatic mutations in the gene that encodes the AR that change the expression level, ligand-binding and other properties of the AR [8–10], and changes in signaling mechanisms that impinge on AR-mediated signal-transduction pathways [11,12]. Despite recent insights into how the AR contributes to disease progression, the precise roles of the AR in PCa, particularly when androgen concentrations are low, are understood poorly.

Nongenomic nuclear-receptor signals

In the classical paradigm, the AR exerts its biological effects by activating the transcription of target genes, a process that is known as the 'genotropic signal'. In addition to the well-established role of steroid hormone receptors as transcription factors, there is considerable experimental evidence that ARs [13–17], estrogen receptor α (ERα) and ERβ [13,18], several types of progesterone receptors (PRs) [19–21], and other members of the nuclear receptor family [22], mediate signaling activities by nongenomic mechanisms [23]. Nongenomic activity is extremely rapid (minutes rather than hours) and not affected by inhibitors of either transcription or protein synthesis such as actinomycin D and cycloheximide. Evidence that steroids exert rapid physiological effects (e.g. some effects of estrogen occur in tissues within seconds) has existed for decades [24]; however, it is only recently that some of the mechanistic aspects of this process became known.

Although some nongenomic effects of nuclear receptor ligands are resistant to nuclear receptor antagonists [25], it is clear that nongenomic steroidal effects can involve classical nuclear receptors [19]. This conclusion arises because rapid actions of steroids have been described that are inhibited completely by well-characterized pharmacological antagonists of the classical receptors [13,18]. In addition, steroid receptors reside in multiprotein complexes in the cytoplasm before ligand binding and nuclear translocation, which allows the possibility of productive interactions with molecules in the cytosol and at extranuclear-membrane surfaces. Because activated, ligand-bound receptors require 0.5–1.0 h to translocate to the nucleus, such interactions are also theoretically possible in the presence of hormone [26].

Cytosolic signal-transduction cascades are activated rapidly by mechanisms that involve classical steroid hormone receptors, and cytoplasmic and membrane effector proteins. 17β-Estradiol (E_2) activates endothelial nitric oxide synthase in vascular endothelial cells within minutes by a mechanism involving ERα phosphoinositide 3-kinase (PI-3K) and the Akt (protein kinase B) serine/threonine kinase [18]. This rapid process, which complements the classical genomic effects of estrogen on vascular pathways [27], might partially explain the cardioprotective effects of estrogen. ERα, but not ERβ, activates signaling from the insulin-like growth factor 1 (IGF-1) receptor by a mechanism that involves direct binding of ERα to the IGF-1 receptor [28]. Recently, membrane ERα has been shown to form signaling complexes with the proto-oncoprotein ErbB2, with subsequent activation of the PI-3K–Akt pathway [29].

Nongenomic androgen signaling

Although currently there is less information on the possible nongenomic roles of the AR than the ERs, nongenomic actions of androgen have been described in at least ten cell types [30]. Some of these involve interactions between ligand and nonclassical receptor systems. Only one AR has been identified to date. However, three isoforms of novel membrane PRs [20,21], a family of seven-transmembrane G-protein-coupled receptors, have been cloned and characterized recently, demonstrating that specialized, membrane-localized steroid receptors might be involved in generating some types of rapid signals. Given the structural similarity between the ligand-binding domains of classical ARs and PRs, it is plausible that novel membrane ARs that belong to this family might exist.

A compelling example of a distinctive, nongenomic signal that is mediated by classical ARs is the recent demonstration that oocyte maturation, which is elicited by steroid hormones in seconds to minutes and does not involve gene transcription, is susceptible to inhibition by classical AR antagonists in *Xenopus* [16,17] and mouse [31]. Although progesterone was thought to be the physiologically relevant steroid in oocyte maturation in *Xenopus*, examination of ovarian extracts following injection of frogs with human chorionic gonadotrophin reveals nearly undetectable levels of progesterone, whereas androstenedione and testosterone are abundant [16]. Thus, studies of steroid effects on oocytes indicate that androgens might be the primary physiological mediator of release from meiotic arrest in oocyte maturation, with the principal hormonal effect activating a nongenomic, rather than a classical genotropic, signaling mechanism. Although the oocyte system is a specialized case, these observations demonstrate the potential for physiologically important androgenic signals that are initiated by either the membrane or cytoplasm.

Another nongenomic pathway has been demonstrated recently using the LNCaP human PCa cell line. Treating these cells with either E_2 or androgen stimulates cell proliferation, which is mediated by the rapid formation of a cytosolic signaling complex containing either ERα or ERβ (depending on cell type), AR and the nonreceptor tyrosine kinase Src [13]. In this study, specific antagonists of ERs and ARs inhibit formation of the complex and downstream signaling, which verifies the participation of the classical steroid receptors in this complex. The SH2 domain of Src interacts with the Tyr-*P* residue at position 537 of ERα, and the SH3 of Src domain interacts with a proline-rich region of the AR. More recently, a scaffolding

protein, named modulator of nongenomic activity of estrogen receptor (MNAR), has been identified as an element of this signaling pathway [32]. Stimulation with E2 forms a ternary complex comprised of ERα, Src and MNAR, which activates Src/mitogen-activated protein (MAP) kinases. MNAR contains multiple LxxLL motifs and two proline-rich regions with multiple PxxP motifs, which mediate interactions with ERα and the SH3 domain of Src, respectively. A recent report suggests that a nongenomic signaling pathway that involves AR, Src and MNAR is upregulated constitutively in androgen-independent LNCaP cells [33]. Further clarification of the rates of formation and the protein components of this and similar cytosolic complexes activated by hormones will provide additional insight into how extranuclear steroid receptors are involved in processing signals that are relevant to tumor-cell behavior.

Although this review deals primarily with nongenomic effects mediated by the classical receptors, there is evidence that nongenomic signals can originate from at least one membrane-associated, non-conventional AR. The most compelling case for this is the demonstration that intracellular Ca^{2+} mobilization in mouse IC21 cells, which are AR-negative and macrophage-like, and splenic T cells, is triggered in response to androgen stimulation [34–36]. Using FITC-labeled, testosterone-coupled bovine serum albumin, Benten and colleagues [34,37] have demonstrated the presence of a surface hormone receptor that can be internalized. Although the Ca^{2+} signal generated in this manner is transient, of low amplitude and does not activate kinase signaling by itself, it is sensitive to toxin inhibitors of G-protein-coupled receptors (GPCRs). This connection to GPCR signaling indicates a potential link with GPR30, the intracellular transmembrane ER that has been identified recently [38].

Targeting androgen receptor to lipid rafts and caveolae

Despite accumulating evidence of signaling events that involve cytosolic, membrane-proximal ARs, the molecular events that govern localization of AR either at or near the plasma membrane and other cell membranes is unclear. One potential mechanism is the direct interaction of AR with an integral membrane component. In support of this, AR was shown recently to be associated with low-density membrane fractions of LNCaP cells that were isolated by sucrose-gradient ultracentrifugation [39]. This study shows that AR interacts directly with the integral membrane protein caveolin-1. Using a mammalian two-hybrid system, AR domains that are required for interaction with caveolin were found to include the N-terminal AF1 domain and the ligand-binding domain. This study of AR is complemented by the finding that ERα also interacts specifically with caveolin-1 [40]. Interaction motifs within ERα have been mapped to two motifs, YNYPEGAAY and FGSNGLGGF, in the N-terminal AF1 domain, which mediate the interaction with the caveolin scaffolding domain (CSD). The CSD has been identified previously in caveolin-interacting molecules that contain either ϕxxϕxxxxϕ or ϕxxxxϕxϕ (in which ϕ represents a hydrophobic residue and x is any residue). Peptides that contain this motif disrupt interactions with caveolin. Interestingly, similar CSD-binding motifs are present in AR (YSWMGLMVFAMGWRSF) and in the mineralocorticoid receptor (FPFMDGSYFSF), but the potential role of these regions in signaling remains to be characterized.

A recent study [41] has demonstrated that palmitoylation of Cys477 in the ligand-binding domain of ERα mediates plasma membrane localization of ERα. A mutant ERα that contains a Cys477Ala substitution is not localized to the cell membrane following ectopic expression in HeLa cells. Significantly, this mutant ERα does not elicit E_2-induced rapid activation of extracellular regulated kinase/MAP kinase signaling. E_2 also reduces both palmitoylation of ERα and its interaction with caveolin in a time- and dose-dependent manner. Although palmitoylation of AR has not been demonstrated, the findings with ERα underscore the relevance of post-translational modifications with membrane-targeting capacity to hormone receptor function. These modifications might provide the opportunity for either permissive or facilitated interactions between nuclear receptor proteins and integral membrane proteins, such as caveolins, which has implications for the regulation of downstream signaling.

Cytosolic androgen receptor and cholesterol-rich lipid rafts

The links between AR and floating fractions in sucrose gradients, and to caveolin-1, that are described above place AR in plasma membrane structures called caveolae and/or cytosolic membrane organelles that communicate with caveolae. Caveolae are an invaginated form of membrane microdomain that are referred to most commonly as 'lipid rafts'. Lipid rafts are cholesterol-rich and sphingolipid-rich components of the plasma membrane that are resistant to extraction with cold nonionic detergents and exhibit light buoyant density in sucrose gradients [42]. Because of their lipid composition, detergent-resistant lipid-raft membranes, which probably comprise ~10% of the plasma membrane area, possess a 'liquid-ordered' structure that distinguishes them from the major, liquid-disordered, fraction of the plasma membrane. Much evidence supports the view that lipid rafts are discrete membrane domains in living cells that perform multiple physiological functions [43–53]. However, difficulties in definition, measuring their size, and the meaning of 'detergent-insolubility' have generated unresolved controversies [42,54–56]. Caveolae, which exhibit an invaginated architecture that is identifiable unambiguously in electron micrographs, were discovered in the 1950s and are the best-studied form of cholesterol-rich raft [46]. The invaginated structure of caveolae results from the presence of one or more members of the caveolin family (caveolin-1, caveolin-2 and caveolin-3) [57]. Caveolins possess an unusual transmembrane structure, and bind cholesterol and many cell-signaling proteins [52].

Lipid rafts are implicated in several signal-transduction mechanisms [43] and other processes, such as cholesterol transport [58] and viral assembly [59]. Because they both sequester and exclude signaling proteins, rafts have been hypothesized to serve as platforms for assembling discrete classes of signaling complexes. Some

signaling proteins, such as heterotrimeric G-protein subunits and Src-like kinases localize preferentially to rafts. A recent review summarizes the published studies that are relevant to a potential role for cholesterol in PCa, with a focus on cholesterol-rich lipid-raft microdomains as potential signaling nodes [60].

Lipid rafts and signal transduction in prostate cancer

Caveolin-1 has been identified as a protein marker that is associated with PCa progression and hormone-refractory disease [61–63]. Evidence indicates that caveolin-1 is a direct mediator of androgen action [39,64], signaling through the PI-3K–Akt pathway [65], and metastasis [64] in PCa. Collectively, these studies indicate a role of caveolar lipid rafts in the signaling mechanisms that are relevant to both androgen-mediated and androgen-independent PCa cell-growth and survival mechanisms.

Although data on caveolin-1 and PCa point to a role for caveolae in PCa progression, lipid rafts also exist in cells that do not contain caveolins [66]. For example, signal transduction by lipid-raft-dependent mechanisms occurs in lymphocytes [44], which do not express caveolins. Although the noncaveolar form of lipid raft is less well-defined than caveolae, the biophysical properties of the two microdomains are similar, if not identical [42]. They also sequester similar classes of proteins [67]. The literature on signal-transduction processes that are mediated by rafts in caveolin-negative cells (e.g. T and B cells [48,68]) indicates that caveolins might not be essential for raft-mediated signaling processes in tumor cells. Despite the fact that caveolin-1 is sometimes overexpressed in prostate and other malignancies, caveolin expression can also be downregulated in malignant cells, and caveolin-1 also has tumor-suppressor-like functions [69,70]. Consequently, it is possible that caveolin-negative rafts might mediate signal-transduction events that affect tumor-cell behavior and/or aggressiveness.

Zhuang *et al.* were the first to show that, in the absence of caveolins, cholesterol-rich lipid-raft microdomains are involved in cell-survival signaling in human PCa cells [71]. In this study, the LNCaP PCa cell line was used to demonstrate a requirement for intact plasma membrane lipid rafts in constitutive and epidermal growth factor receptor (EGFR)-stimulated signaling through the Akt serine/threonine kinase. Disrupting rafts using cholesterol-binding compounds inhibits both EGFR and Akt signaling and induces apoptosis. These effects are reversed by restoring cholesterol to the plasma membrane, indicating that both the signal transduction and cell-survival effects of cholesterol-binding agents are mediated by a cholesterol-dependent process. Survival of LNCaP cells requires continuous signaling through the PI-3K–Akt pathway [72], which is most likely to mimic the situation in many human cancers. The dependence of LNCaP cells on Akt-mediated signaling for survival seems to be a property of aggressive PCa cells [73,74] and androgen ablation upregulates this pathway in model systems [75]. The PI-3K–Akt–mTOR pathway is now a major focus for the design of novel drugs with potential clinical application [76,77].

Another recent study by Kim *et al.* demonstrates a role of caveolin-negative lipid rafts in interleukin 6 (IL-6)–signal transducer and activator of transcription 3 (STAT3)-mediated neuroendocrine differentiation of LNCaP cells [78]. This study shows that phosphorylation of STAT3 by IL-6 and cytokine-stimulated translocation of STAT3 to the nucleus are inhibited by the cholesterol-binding drug filipin. Isolation of Triton X-100-insoluble raft fractions by sucrose gradient ultracentrifugation demonstrates that the 80 kDa IL-6 receptor localizes almost exclusively to the raft membrane fraction. Similarly, STAT3 in the raft fraction is phosphorylated preferentially by IL-6. The IL-6-stimulated increase in expression of neuroendocrine markers is also inhibited by filipin. This study also demonstrated that LNCaP cells do not contain caveolins. Therefore, raft-dependent signaling mechanisms do not require caveolins to elicit physiologically relevant signals in cancer cells. Importantly, IL-6 [79] and STAT3 [80] are marker proteins for PCa that can affect signal transduction through the AR [11,81], and neuroendocrine differentiation might be a marker of aggressive disease in PCa as in other solid tumors [82].

Zhuang *et al.* [71] and Kim *et al.* [78] suggest that two, independent signaling mechanisms that are relevant to clinical PCa (PI-3K–Akt and IL-6–STAT3) are mediated, at least in part, by raft-dependent mechanisms. Thus, studies of the raft compartment might provide new insights into the biochemical basis of growth and survival signals in PCa and other solid tumors. If true, what is the role of the AR in raft-dependent signaling?

Membrane rafts as privileged sites for nongenomic signaling

Two groups have reported recently that treating AR-expressing cells with an androgen rapidly activates PI-3K–Akt signaling [14,15], most likely by direct binding of ARs to PI-3K subunits. This links a nongenomic androgen signal to a parallel signal-transduction pathway under the control of lipid rafts [71]. AR localizes to the caveolar form of raft [39] and non-caveolar (flat) rafts (B. Cinar and M.R. Freeman, unpublished) (Figure 1). Additional unpublished evidence from our group shows that androgens enhance the localization of AR to rafts, which indicates that the presence of ARs in rafts is regulated. We also have evidence that the AR forms complexes with other signaling complexes in rafts that are isolated from LNCaP cells. Both caveolar rafts and undifferentiated flat rafts are crucial nodes for signal transduction in several cell types. The observation that ARs can transit and/or reside stably in membrane rafts indicates that the AR functions as a signaling intermediate in this subcellular location. Several signaling pathways, including IL-6, IL-4, IGF and ErbB receptor mechanisms, interact with the AR pathway [83], and it remains to be determined whether these intersect with the AR exclusively via the classical genotropic pathway. The many known binding partners of the AR [84], most of which have no clear regulatory function for the AR, also indicate that the AR might participate directly in productive, nongenomic signaling processes.

Rafts can be isolated biochemically [39,78,85] and visualized using imaging techniques [86,87]. These and

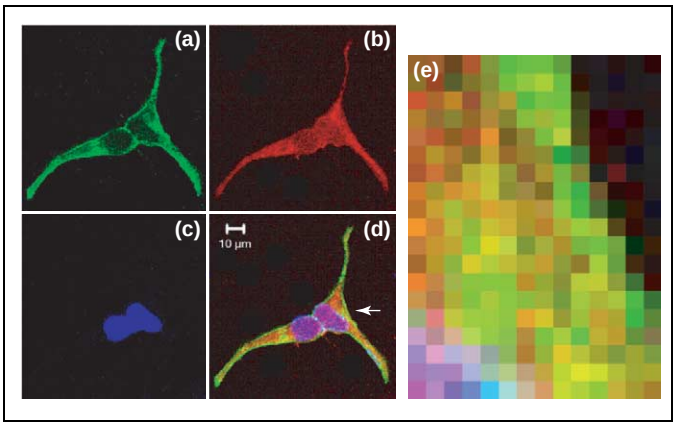

Figure 1. Co-localization of ARs and lipid rafts in caveolin-negative LNCaP cells. **(a)** Multivalent FITC–cholera toxin B conjugate is used to patch and stain the raft ganglioside marker GM1 (green). **(b)** AR is detected using Texas-Red-conjugated anti-mouse secondary antibody (red). **(c)** Nuclei (blue) are stained with DAPI. **(d)** Co-localization of AR and GM1 (yellow) is evident in the merged images. **(e)** Magnified region, indicated by arrow in (d).

other methods allow a focused approach to unraveling the mechanism(s) of nongenomic AR action by testing the hypothesis that the lipid-raft-membrane compartment is a privileged site of nongenomic nuclear-receptor signaling (Figure 2). In this model, hormone binding activates ARs, which causes most ARs to translocate to the nucleus, which results in activation of the genotropic signal. However, a minority of the cytosolic AR proteins might also transit to either caveolae or to flat, caveolin-negative rafts (B. Cinar and M.R. Freeman, unpublished) where productive interactions with other signaling proteins might occur. These might affect cellular physiology in ways that are relevant to tumor-cell behavior *in vivo*, such as growth, resistance to apoptotic signals and motility.

The hypothesis that ARs in rafts signal independently of transcription can be tested directly by: (i) high-resolution analysis of the dynamic behavior of AR under conditions of hormonal activation and in response to other AR-pathway activators, such as soluble growth factors and cytokines; (ii) isolation and characterization of signaling complexes that contain AR from the non-nuclear subcellular spaces; and (iii) analyzing signals that are triggered by genomically inactivated AR. Because of the tendency of the raft compartment(s) to sequester signal-transduction complexes, we believe this subcellular location represents a rich, potential source of information on the role of the AR in nongenomic signaling.

Concluding remarks

There is extensive evidence that nuclear receptors are active in cytosolic locations and in association with membrane-resident protein complexes. This review provides a rationale for focusing on cholesterol-rich membrane rafts as potential sites of processing of nongenomic signals that involve the AR and other classical steroid receptors. Although the significance of nongenomic actions of steroid hormones in cancer is controversial and debated actively, the well-known loss of hormonal control of the AR during PCa progression provides a fertile, clinically relevant area for investigating these potentially important signaling mechanisms.

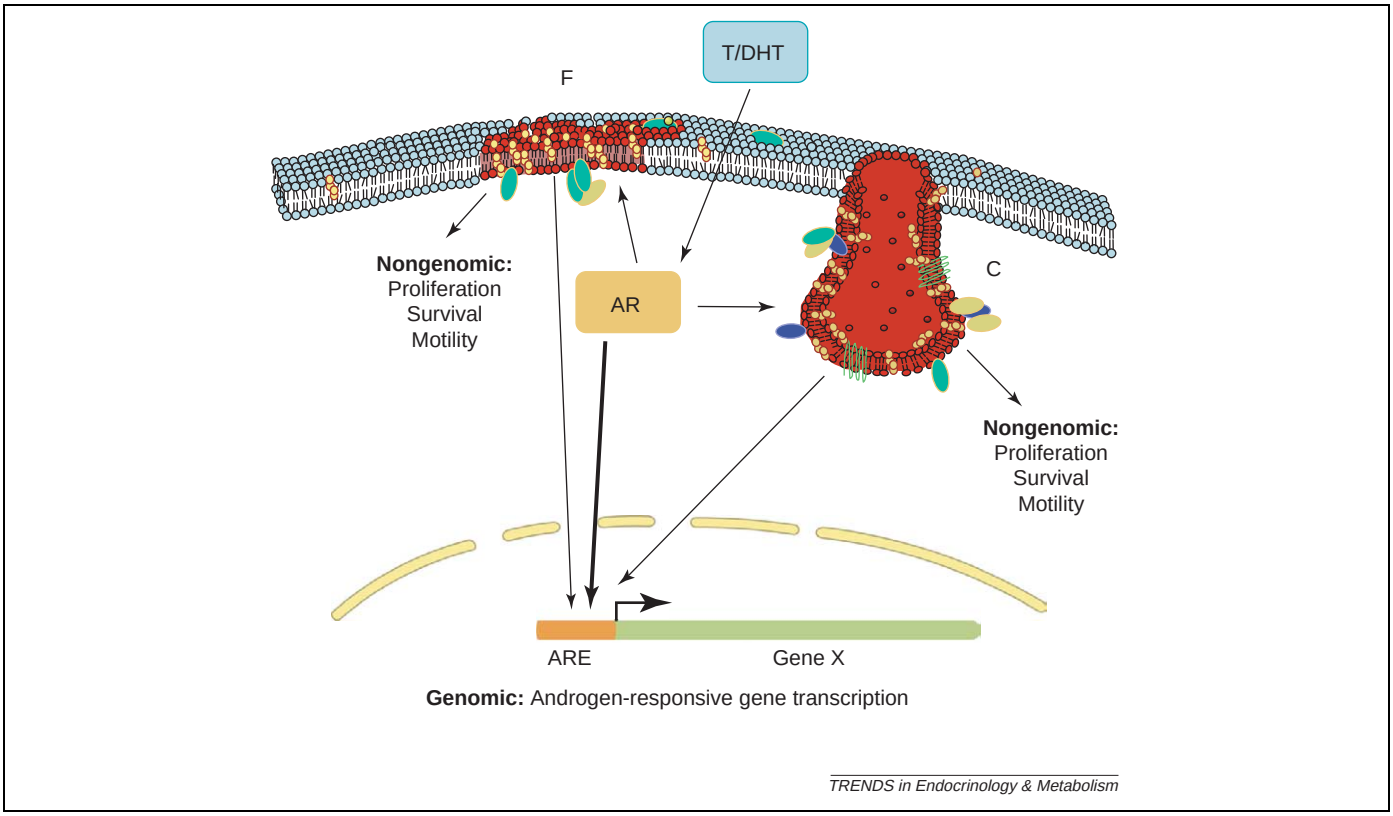

Figure 2. Androgen signaling by cholesterol-rich lipid rafts. AR-mediated nongenomic signals might arise by a mechanism that involves transit of the AR through either flat (F) or caveolar (C) membrane rafts. This mechanism is distinct from the genomic signal (bold arrow). Raft-mediated signals might also impinge on androgen-regulated genes in PCa cells through processes that involve ARs in raft domains. The nongenomic biological activities illustrated are hypothetical. Abbreviations: ARE, androgen-response element; T/DHT, testosterone/dihydrotestosterone.

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